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(54) Title: METHODS AND COMPOSITIONS FOR CONTROLLING BIOFILM DEVELOPMENT			
(57) Abstract A method of cleaning or protecting surfaces by treatment with compositions comprising N-(3-oxododecanoyl)-L-homoserine lactone (OodHLL) blocking compounds and/or N-butyryl-L-homoserine lactone (BHL) analogs, either in combination or separately.			

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METHODS AND COMPOSITIONS FOR
CONTROLLING BIOFILM DEVELOPMENT

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of the filing date of Provisional Application 60/050,093, filed June 18, 1997.

5 STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

TECHNICAL FIELD

10 This invention relates to certain homoserine lactone compositions and methods for their use in biofilm regulation.

BACKGROUND OF THE INVENTION

15 Biofilms are biological films that develop and persist at interfaces in aqueous environments (Geesey, *et al.*, Can. J. Microbiol. 32, 1733-6, 1977; 1994; Boivin and Costerton, Elsevier Appl. Sci., London, 53-62, 1991; Khoury, *et al.*, ASAIO, 38, M174-178, 1992; Costerton, *et al.*, J. Bacteriol., 176, 2137-2142, 1994), especially along the inner walls of conduit material in industrial
20 facilities, in household plumbing systems, on medical implants, or as foci of chronic infections. These biological films are composed of microorganisms embedded in an organic gelatinous structure composed of one or more matrix polymers which are secreted by the resident
25 microorganisms. Biofilms can develop into macroscopic

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structures several millimeters or centimeters in thickness and can cover large surface areas. These biological formations can play a role in restricting or entirely blocking flow in plumbing systems and often decrease the life of materials through corrosive action mediated by the embedded bacteria. Biofilms are also capable of trapping nutrients and particulates that can contribute to their enhanced development and stability.

The involvement of extracellular polymers in bacterial biofilms has been documented for both aquatic (Jones, *et al.*, J. Bacteriol., 99, 316-325, 1969; and marine bacteria (Floodgate, Memorie dell' Instituto italiano di idrobiologica Dott. Carco di Marchi 29 (suppl.), 309-323 1972), and the association of exopolysaccharides with attached bacteria has been demonstrated using electron microscopy (Geesey, *et al.*, supra; Dempsey, Marine Biol. 61, 305-315, 1981) and light microscopy (Zobell, J. Bacteriol. 46, 39-56, 1943. The presence of such exopolysaccharides is believed to be involved in the development of the microbial biofilm (Allison and Sutherland, J. Microbiol. Methods, 2, 93-99, 1987). Analysis of biofilm bacteria isolated from freshwater and marine environments has shown that the polymers they produce are composed largely of acidic polysaccharides. The control and removal of biofilm material from pipe and conduit surfaces has historically been carried out by the addition of corrosive chemicals such as chlorine or strong alkali solutions or through mechanical means. Such treatments are generally harsh to both the plumbing systems and the environment, and have been necessary due to the recalcitrant nature of biofilms within those systems. The resistance to treatment by biocides has been due in large measure to the protective character of intact biofilm matrix polymers (Srinivasan, *et al.*, Biotech. Bioeng., 46, 553-560, 1995.

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Modern methods of direct observation of living biofilms have established the very complicated structural architecture (Costerton, et al., Ann., Rev. Microbiol., 49, 711-745, 1995) of these sessile microbial populations. The overriding structural element responsible for development and maintenance of this biofilm architecture is the matrix polymer that is produced by the resident microorganisms. That populations of associated bacteria could produce structures as complex as are found in biofilms suggested the operation of a cell-cell signaling mechanism.

Prior to 1981, microbiologists had generally assumed that bacteria had neither the requirement nor the capability of producing cell-cell signaling molecules. In 1981, it was shown by Eberhard, et al. Biochemistry, 20, 2444-2499, 1981, that the bacterium *Photobacterium fischeri* produces a compound 3-oxo-N-(tetrahydro-2-oxo-3-furanyl) hexanamide, also known as vibrio (photobacterium) autoinducer (VAI), which is associated with bacterial luminescence under conditions of high cell density. The cell membrane of *P. fischeri* was shown to be permeable to VAI by Kaplan and Greenberg in 1985 (J. Bacteriol., 163, 1210-1214, 1985). At low bacterial cell densities in broth medium, VAI passively diffuses out of the cells along a concentration gradient, where it accumulates in the surrounding medium. At high cell densities the concentration of VAI outside the cells is equivalent to the concentration of VAI inside the cells. Under such conditions VAI was shown to initiate transcription of luminescence genes. Using such a system, bacteria are able to monitor their own population density and regulate the activity of specific genes at the population level.

For several years it was presumed that the autoinducer involved in bacterial luminescence was unique to the few bacteria that produce light in the marine

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environment. Then, in 1992, the terrestrial bacterium *Erwinia carotovora* was shown to use an autoinducer system to regulate the production of the β -lactam antibiotic carbapenem (Bainton, et al., Biochem J., 288, 297-1004, 1992b). The molecule found to be responsible for autoinduction of carbapenem was shown to be an acylated homoserine lactone (HSL), a member of the same class of molecule responsible for autoinduction in bioluminescence. This finding led to a general search for HSLs in a wide range of bacteria. To affect the search, a bioluminescence sensor system was developed and used to screen for HSL production in the spent supernatant liquids of a number of bacterial cultures. Many different organisms were shown by the screening to produce HSLs. These included: *Pseudomonas aeruginosa*, *Serratia marcescens*, *Erwinia herbicola*, *Citrobacter freundii*, *Enterobacter agglomerans* and *Proteus mirabilis* (Brainton, et al., Gene. 116, 87-91, 1992a; Swift, et al., Mol. Microbiol., 10, 511-520, 1993). More recently, the list has grown to include *Erwinia stewartii* (Beck, J. Bacteriol, 177, 5000-5008, 1993), *Yersinia enterocolitica* (Throup, et al., Mol. Microbiol., 17, 345-356, 1995), *Agrobacterium tumefaciens* (Zhang, et al., Nature, 362, 446-448, 1993), *Chromobacterium violaceum* (Winston, et al., Proc. Natl. Acad. Sci., USA, 92, 9427-9431, 1995), *Rhizobium leguminosarium* (Schripsema, et al., J. Bacteriol, 178, 366-371 1996) and others. Today it is generally assumed that all enteric bacteria, and the gram negative bacteria generally, are capable of cell density regulation using HSL autoinducers.

In 1993 Gambello, et al. Infect. Immun., 61, 1880-1184, (1993) showed that the α -HSL product of the LasI gene of *Pseudomonas aeruginosa* controls the production of exotoxin A, and of other virulence factors, in a cell density dependent manner. Since that time, the production of a large number of *Pseudomonas* virulence factors have

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5 been shown to be controlled by α -HSL compounds produced by the LasI and RhlI regulatory systems (Ochsner, et al., Proc. Natl. Acad. Sci., USA 92, 6424-6428, 1995; Winson, et al., supra; Latifi, et al., 1995), in a manner reminiscent of the Lux system. Latifi, et al. Mol. Microbiol, 21, 1173-1146, (1996) have also shown that many stationary phase properties of *P. aeruginosa*, including those controlled by the stationary phase sigma factor (RpoS), are under the hierarchical control of the LasI and RhlI cell-cell signaling systems.

10 In all cases, homoserine lactone autoinducers are known to bind to a DNA binding protein homologous to LuxR in *Photobacterium fischeri*, causing a conformational change in the protein initiating transcriptional activation. This process couples the expression of specific genes to bacterial cell density (Latifi, et al. supra, 1996). Regulation of this type has been called 'quorum sensing' because it suggests the requirement for a 'quorate' population of bacterial cells prior to activation of the target genes (Fuqua, et al., J. Bacteriol., 176, 269-275, 1994b). Expression of certain of these 'virulence factors' has been correlated with bacterial cell density (Finley and Falkow, Microbiol. Rev. 53, 210-230, 1989).

25 In *P. aeruginosa*, quorum sensing has been shown to be involved in the regulation of a large number of exoproducts including elastase, alkaline protease, LasA protease, hemolysin, cyanide, pyocyanin and rhamnolipid (Gambello, et al., supra; Latifi, et al., supra; Winson, et al., supra; Ochsner, et al., 1995); but has never before been shown to be involved in biofilm formation. Most of these exoproducts are synthesized and exported maximally as *P. aeruginosa* enters stationary phase.

30 The concept of cell signalling and quorum sensing has been studied in the art. See for example U.S. Patent No. 5,591,872, to Pearson et al; Passador et al, Journal of

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Bacteriology, pages 5990-6000, October, 1996, PCT WO92/18614 and U.S. Patent No. 5,593,827.

However, none of these prior publications recognize how these principles may be applied to enable the regulation of biofilms according to this invention.

BRIEF SUMMARY OF THE INVENTION

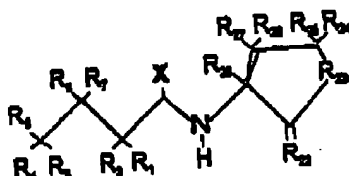
It is accordingly one object of the present invention to provide compositions and methods for the regulation of biofilms.

A further object of the invention is to provide novel methods for regulation, i.e., inhibition, enhancement, dispersion, etc., by administration of a group of homoserine lactone blockers or analogs.

An even further object of the invention is to provide methods to control, enhance, stimulate, diminish, prevent, inhibit, detach, remove, clean, and/or disperse biofilm development by administration of a selected group of homoserine lactone blockers or analogs.

Other objects and advantages of the invention will become apparent to those skilled in the art as the description of the invention proceeds.

In satisfaction of the foregoing objects and advantages, the present invention provides compositions to control, enhance, stimulate, diminish, prevent, inhibit, detach, remove, clean, and/or disperse biofilms which comprises a composition having as its active ingredient one or more homoserine lactone blocker or analog compounds. Especially preferred blockers and/or analogs, are selected from those of the following formulae:

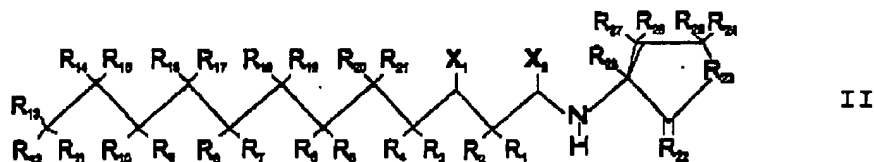


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and



wherein in the above formulae R_1 - R_{22} are selected from H, C_1 - C_4 alkyl group (preferably CH_3), OH, NH_2 , SH or a halogen such as fluorine, chlorine bromine or iodine;

R_{22} and R_{23} are selected from S, O, and N-R,

R_{24} - R_{28} is H or a halogen, and

X, X_1 and X_2 are selected from O, S, H_2 or any combination of H plus one halogen or two halogens when one or more R groups is substituted.

The present invention also provides methods to control, enhance, stimulate, diminish, prevent, inhibit, detach, remove, clean, and/or disperse biofilms, which method comprises administration of a composition of the invention. Included in such methods are methods to remove biofilm from surfaces to effect cleaning of the surfaces.

BRIEF DESCRIPTION OF THE FIGURES

Reference is now made to the drawings accompanying the application wherein:

Figure 1 is a "Nearest Neighbor Analysis" which shows the average distance between cells in biofilms composed of wild-type *P. aeruginosa* PAO1, the *lasI/rhlI* knockout mutant *P. aeruginosa* PAO-JP2, the *LasI* knockout *P. aeruginosa* PAO-JP1, the *rhlI* knockout *P. aeruginosa* PDO100 and *P. aeruginosa* JP1 grown in the presence of exogenous OddHL N-(3-oxododecanoyl)-L-homoserine lactone (10 μ M);

Figures 2a, 2b, 2c, and 2d are cell cluster studies which show the effect of the addition of 0.2% SDS to a biofilm cell cluster of the *lasI* knockout strain *P.*

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aeruginosa PAO-JP1. The strain was grown in EPRI medium for 10 days in continuous culture reactor under flowing conditions (a). SDS was added to the culture reactor and flow was turned off for 30 min (b,c). Following incubation in the presence of SDS, flow was reinitiated (d);

Figures 3a and 3b are alginate promoter studies which show the activity of the alginate promoters *algC*, and *algD*. Cultures of *P. aeruginosa* strain 8830 were grown for 48 hours in batch culture flasks containing EPRI medium. Test flasks were amended by either 10 μ M OddHL (FAI), 20 μ M BHL (butyryl-L-homoserine lactone) or both (FAI + BHL). Following incubation, cells were harvested and promoter activity was measured as specific activity of the beta-galactosidase reporter enzyme as normalized to cell protein. The top graph (Fig. 3a) shows the results for *algC* specific reporter activity. The bottom graph (Fig. 3b) shows the results for *algD* specific reporter activity;

Figures 4a, 4b, 4c, 4d, 4e and 4f is a comparison study which shows the effect of 0.2% SDS on wild-type *P. aeruginosa* PAO1. The bacteria were grown in EPRI medium in continuous culture for 10 days (a,d). Following the incubation period, 0.2% SDS was added to the reactor and flow was halted for 30 minutes (b,e). Following incubation with SDS, flow was reinitiated (c,f). The left panel shows that during such treatment, wild-type PAO1 (untreated) shows no indication of cell dispersion (a,b,c). The right panel shows that when the wild-type strain is cultured in the presence of 20 μ M OddHL analog (N-(2,2-difluorodecanoyl-L-homoserine lactone), the cell cluster disperses completely following reinitiation of flow (d,e,f).

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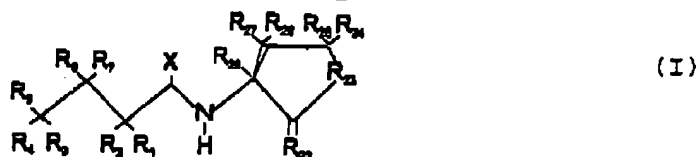
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DESCRIPTION OF THE INVENTION

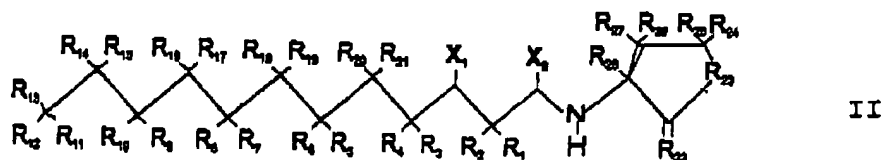
As noted above, the present invention provides compositions and methods for biofilm regulation as exemplified by biofilm removal from surfaces, for example hard and soft surfaces, woven and non-woven surfaces, and porous and non-porous surfaces. Other examples of surfaces which may be cleaned by removal of biofilm therefrom using the compositions of the invention include toilet bowls, bath tubs, drains, chairs, counter tops (such as those exposed to meats such as chicken), vegetables, meat processing rooms, butcher shops, airducts, air conditioners, carpets, paper or woven product treatment, diapers, healthy air machines, and in other areas as described herein.

The preferred compositions of the invention comprise active blocker or analog compounds selected from the group consisting of one or more compounds of the formulae:

N-Butyryl-L-Homoserine Lactone Analogs:



N(3-oxododecanoyl)-L-homoserine Lactone Blockers:



wherein in the above formulae R_1 - R_{21} are selected from H, C_1 - C_4 alkyl group (preferably CH_3), OH, NH_2 , SH or a halogen such as fluorine, chlorine bromine or iodine;

R_{22} and R_{23} are selected from S and O,

R_{24} - R_{25} is H or a halogen, and

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X, X₁ and X₂ are selected from O, S, H₂ or any combination of H plus one halogen or two halogens when one or more R groups is substituted.

5 The compounds of formulae (I) and (II) are fully disclosed and described in copending United States Application Serial No. _____, filed June 18, 1998, (Attorney Docket No. 50198-099).

10 While the compositions and methods of the invention preferably employ active blocker and analog compounds as described herein, it should be understood that other active blockers and analogs may be used if they comply with the criteria described below.

15 The biofilm removing composition of this invention may be in the form of the active compound and a vehicle or carrier such as water or non-aqueous vehicle. Aqueous solutions or suspensions containing an effective amount of the active compound suitable to control, enhance, stimulate, diminish, prevent, inhibit, detach, disperse, remove and/or clean a biofilm on a surface, are preferred.

20 The cleaning composition may also be in the form of a spray, a dispensable liquid, or a toilet tank drop-in or rim block for prevention and removal of soil and under-rim cleaning for toilets.

25 The methods of the present invention comprise administering a cleaning-effective amount of the active composition described above to a biofilm-containing surface. In another embodiment of the invention, the method comprises the step of administering a cleaning-effective amount of the composition to a surface which

30 will prevent normal biofilm development.

As described herein, the discovery that cell communication molecules appear to be involved in biofilm development, has led to the present invention, and is the basis for the novel compositions and methods of the

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invention. At least two known N-acyl-L homoserine lactones appear responsible for the regulation of *Pseudomonas aeruginosa* biofilms; these are N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and N-butyryl-L-homoserine lactone (BHL). The former has been demonstrated to regulate the development of *Pseudomonas aeruginosa* biofilms and to be responsible for maintaining the integrity of biofilm structures by controlling the production of biofilm matrix polymers. The latter has been shown to be involved in the dispersion of *Pseudomonas aeruginosa* biofilms, and regulating the production and release of molecules responsible for breaking down biofilm matrix material. These homoserine lactones have been isolated from a wide range of bacteria, and it is believed that they are also responsible for biofilm regulation in organisms other than *P. aeruginosa*.

By artificially manipulating the binding of homoserine lactones to their cognate receptor molecules, according to the present invention one can control the formation, development, persistence and dispersion of microbial biofilms. For instance, the addition of a blocking compound which blocks the binding of OdDHL to its cognate receptor (LasR) prevents the production of matrix polymer material as the bacteria continue to multiply. The result is that cell aggregates formed under these conditions can be easily dispersed by the addition of simple surfactants. Additionally, developed biofilms can be treated with an analog of the homoserine lactone BHL to induce the release of enzymes which can digest the biofilm matrix material and disperse the bacteria into the bulk medium. Such treatments can be used as an effective means of controlling biofilm ecology in nature and in cleaning applications.

The biofilm cleaning and removal compositions of the present invention comprise an active compound selected from the group consisting of OdDHL blocker compounds, and

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analogs of BHL, and especially one or more compounds of formula (I) or (II) described herein. By blocker compounds is meant a compound that will prevent native OdDHL from binding to its receptor protein (LasR).
5 Blocker compounds could competitively bind to the OdDHL receptor or could bind to OdDHL itself to render it incapable of binding.

By analog is meant a compound that will mimic the activity of BHL. Most preferably, an analog of the
10 present invention will bind to the BHL receptor in an irreversible manner.

Compounds which are blockers for OdDHL are functionally defined as any compound that interferes with normal biofilm development by interfering with the natural
15 activity of OdDHL. Normal biofilm development is determined by measuring the following parameters in a microbial community at an interface: 1) Cell to cell distance, 2) Cell cluster thickness, 3) Alginate or other matrix polymer production, 4) Susceptibility to dispersion
20 by surfactant. These parameters are defined more fully below under the heading: "Methods to verify the suitability of candidate compounds". Any compound which influences one or all of the above parameters by interfering with the activity of natural OdDHL shall be
25 considered a blocking analog for OdDHL activity.

Compounds which are analogs of BHL are functionally defined as any compound that either mimics or enhances the natural activity of BHL as its function relates to biofilm dispersion. The natural activity of BHL is defined
30 operationally as stimulating the release of enzymes from bacteria which are responsible for the breakdown of biofilm matrix polymers. This breakdown is measured by the reduction of biofilm extracellular polymers to smaller molecular weight fractions and/or by observable sloughing
35 or dispersion of a biofilm following the release of such enzymes.

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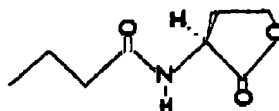
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Due to the simple nature of the homoserine lactones as a group, analogs and blockers may be produced that can act on *P. aeruginosa* and other bacteria and microorganisms in general, particularly on pseudomonads and gram negative bacteria.

The two known homoserine lactones mentioned above are depicted below:

N-Butyryl-L-Homoserine Lactone (BHL)

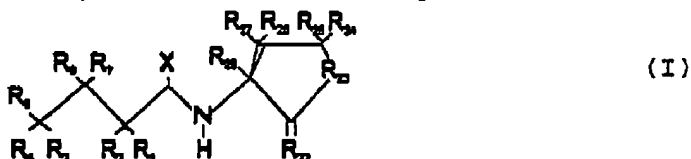


N(3-oxododecanoyl)-L-homoserine Lactone (OdDHL)

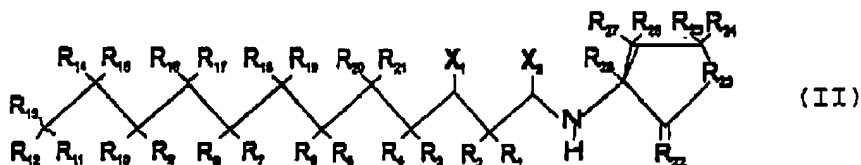


The preferred OdDHL blockers and BHL analogs of this invention are most preferably selected from the compounds disclosed below:

N-Butyryl-L-Homoserine Lactone analogs:



N(3-oxododecanoyl)-L-homoserine Lactone blockers:



wherein, in the above formulae, R_1 - R_{21} are selected from H, C_1 - C_4 alkyl group, (preferably CH_3), OH, NH_2 , SH or a halogen such as fluorine, chlorine bromine or iodine; R_{22} and R_{23} are selected from S and O; R_{24} - R_{28} is H or a halogen; and

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X, X₁ and X₂ are selected from O, S, H, or any combination of H plus one halogen or two halogens when one or more R group is substituted.

5 The preceding are preferred analog and blocker structures for homoserine lactones of the invention. A successful blocker will competitively bind to the cognate receptor protein (LasR) and block the binding of OdDHL, either inducing the receptor protein to bind to its receptor sites on the DNA molecule or preventing the
10 receptor Protein from binding to its receptor sites on the DNA molecule.

These blockers and analogs may be modified without departing from the scope of the invention. Modifications may be as follows:

- 15 1) Alteration of the acyl side chain by increasing or decreasing its length.
- 2) Alteration of the structure of the acyl side chain, such as addition of a double bond or a triple bond between carbon atoms within the acyl side chain.
- 20 3) Substitution on carbons in the acyl side chain, e.g., the addition of a methyl group or other group such as an oxo-group, a hydroxyl group, an amino group, a sulfur atom, a halogen or dihalogen or some other atom or R-group to any location along the acyl side chain.
- 25 4) Substitution of carbons comprising the backbone of the acyl side chain with S or S substituted moieties.
- 5) Substitution on the homoserine lactone ring portion of the molecule. For example: addition of a sulfur group to produce a thiolactone.
- 30 6) Halogenated acyl furanones have been shown to act as blockers to homoserine lactone cognate receptor proteins.
- 7) Ring size of the acryl side chain varying heterocyclic moiety is variable. For example, 4-membered and 6-membered rings containing nitrogen (i.e., beta and
35 delta lactoams) are included.

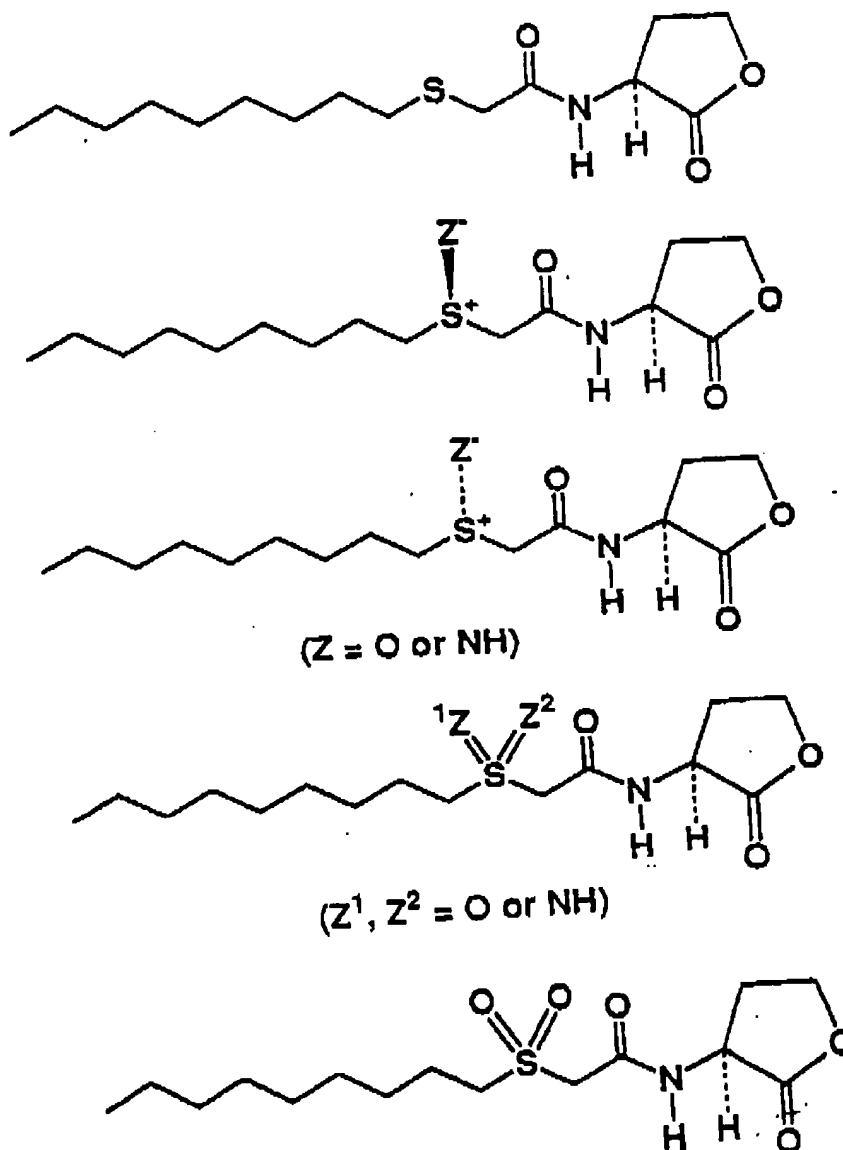
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Also, blocking analogs may bind to the HSL and scavenge it in free form from the environment. Such analogs have a binding site to OdDHL with a similar structure but greater affinity than the cognate binding protein, LasR.

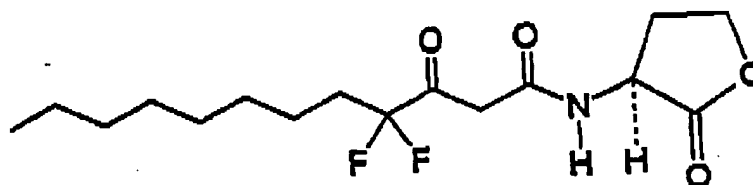
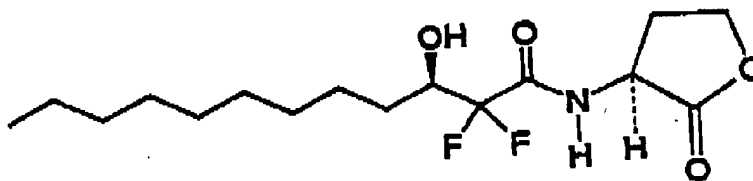
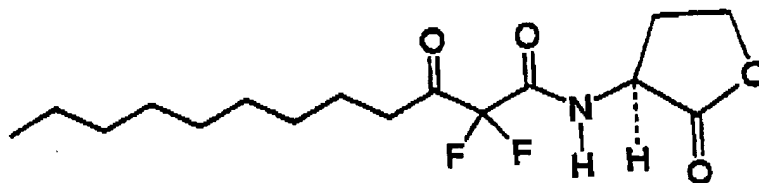
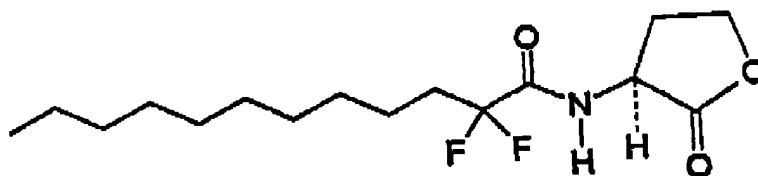
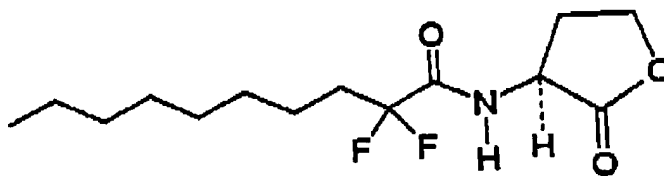
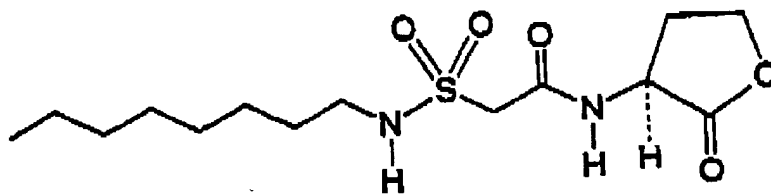
The following are specifically preferred fluorinated and sulfur substituted blockers and analogs for use in the invention:



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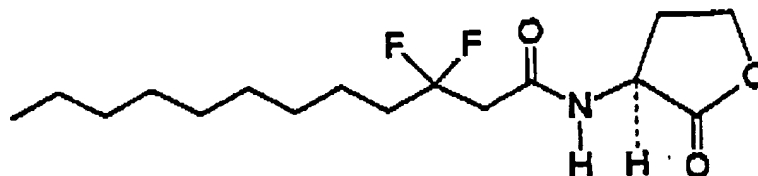
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The present invention is suitable for biofilms originating from a single type of organism and for mixed biofilms. By "mixed biofilms" is meant biofilms created by more than one type of microorganism. Most preferably, it is envisioned that biofilms will be created by at least two organisms from the group consisting of bacteria, algae, fungi, and protozoa.

The effects of treating biofilms with homoserine lactones have been demonstrated with *Pseudomonas aeruginosa*. The HSLs have generally been isolated from a wide range of bacteria known to produce biofilms. Among these are the enterobacteria. The presence of the HSLs in a wide range of bacteria indicates that the compounds of the present invention can be used to effectively treat not only *Pseudomonas* sp. biofilms but also mixed biofilms containing *Pseudomonas* sp. and biofilms composed of bacteria other than *Pseudomonas aeruginosa*.

The following is a list of groups of Gram-Negative bacteria that have members which use homoserine lactones for cell-cell communication: anaerobic Gram Negative Straight, Curved and Helical Rods; *Bacteroidaceae*; The *Rickettsias* and *Chlamydias*; Dissimilatory Sulfate - or Sulfur-Reducing Bacteria; the *Mycoplasmas*; The *mycobacteria*; Budding and/or Appendaged Bacteria; Sheathed Bacteria; *Nocardioforms*; and *Actinomycetes*, for example. See Bergey's Manual of Systematic Bacteriology, First Ed., John G. Holt, Editor in Chief (1984), incorporated herein by reference.

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Compositions of the present invention may be in the form of an aqueous solution or suspension containing a cleaning-effective or prevention effective amount of the active compound described above. The cleaning composition
5 may be in the form of a spray, a dispensable liquid, or a toilet tank drop-in under-rim product for prevention, removal and cleaning of toilets and other wet or intermittently wet surfaces in domestic or industrial environments.

10 The compositions of the present invention preferably additionally comprise a surfactant selected from the group consisting of anionic, nonionic, amphoteric, biological surfactants and mixtures thereof. Most preferably, the surfactant is sodium dodecyl sulfate. The surfactant
15 should be present in an amount at least 0.2% by weight, with a range of from 0.1% to 10% by weight.

One may wish to add other efficacious adjuvant compounds to the cleaning solution of the present invention. Most preferably, one may wish to add biocides,
20 fungicides, antibiotics, and mixtures thereof to affect planktonics. One may also wish to add pH regulators, perfumes, dyes or colorants.

By "cleaning-effective" amount of active compound, it is meant an amount of the compound which is necessary
25 to remove at least 10% of bacteria from a biofilm as determined by a reduction in numbers of bacteria within the biofilm when compared with a biofilm not exposed to the active compound.

By "prevention-effective" amount of active compound, it is meant an amount of compound that is effective to
30 prevent normal biofilm formation between normal cleaning times, most preferably preventing any biofilm build-up as determined by a statistically significant increase in the number of cells within a biofilm or upon a clean surface.
35 Prevention of normal biofilm formation is determined by the ability to disperse a biofilm using surfactants and

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or detergents or other chemical treatments which will result in the removal of bacterial cells from a biofilm. To distinguish between normal biofilm formation and treatment with a prevention-effective amount of active compound, the treated biofilm must be shown to release 10% or more of its bacteria when compared with a similar biofilm that is not treated when both biofilms are exposed to surfactants and or detergents or other chemical treatments which will result in the removal of bacterial cells from a biofilm.

The application of OddHL blocker to a solution should be in the concentration range of 10 nM to 100 uM and is preferably dissolved in a carrier containing a 3 to 1 ratio of water and ethanol or other organic solvent by volume. Further, the OddHL blocker can be impregnated into a surface or other solid or semi-solid carrier which would release the blocker into the bulk environment at a concentration of 10 nM to 100 uM.

The application of BHL analog to a solution should be in the concentration range of 20 nM to 200 uM and is preferably dissolved in an aqueous carrier. Further, BHL or a BHL analog can be impregnated into a surface or other solid or semi-solid carrier which would release the compound into the bulk environment at a concentration of 20 nM to 200 uM.

METHODS OF THE PRESENT INVENTION

In another embodiment, the present invention is a method of biofilm removal or prevention. This method is suitable for both cleaning and preventing biofilm deposits and preventing biofilm deposits from accruing. It is envisioned that one would treat hard, rigid surfaces such as drain pipes, glazed ceramic, porcelain, glass, metal, wood, chrome, plastic, vinyl and formica or soft flexible surfaces such as shower curtains, upholstery, laundry and carpeting. It is also envisioned that both woven and non-

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woven and porous and non-porous surfaces would be suitable.

5 In one embodiment, the method comprises the steps of administering a cleaning-effective amount of the cleaning compound described above to a biofilm-containing surface or a surface that one wishes to remain biofilm-free. In another form of the present invention, one would administer an amount of the cleaning compound described above effective to prevent biofilm build-up or formation
10 on a surface.

In particularly advantageous forms of the present invention, the method is used to remove biofilm on food preparation surfaces, such as kitchen counters, cutting boards, sinks, stoves, refrigerator surfaces, or on
15 sponges and other cleaning implements, such as mops and wipes.

In another advantageous form of the present invention, the method is used to remove biofilm on bathroom surfaces, such as toilets, sinks, bathtubs, showers, and drains.
20

In another form, the present invention is used to remove biofilm on clothing and other woven and soft surfaces. This may be by means of a wipe, sponging or soaking method or by a laundering or detergent method.
25 In another form of the present invention, the method is used to remove biofilm on floors and window surfaces, especially surfaces that are exposed to moisture, such as kitchen floor, shower stalls, and food production areas. In another form of the present invention, the method is
30 used to remove biofilm in large-scale sanitation applications, such as food production machinery, processing areas and conduits that carry raw materials or finished products.

In other forms of the present invention, the method
35 is used to prevent or remove biofilm as a dentifrice, a mouthwash, a compound for the treatment of dental caries,

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acne treatment, cleaning and disinfecting contact lenses, and medically implanted devices that are permanent such as an artificial heart valve or hip joint, and those that are not permanent such as indwelling catheters, pacemakers, surgical pins etc. The method is further used to prevent or remove biofilm in situations involving bacterial infection of a host, either human or animal, for example in a topical dressing for burn patients. An example of such a situation would be the infection by *P. aeruginosa* of superficial wounds such as are found in burn patients or in the lung of a cystic fibrosis patient.

In other forms, the present invention can be used to control or prevent the development of biofilm in the process of manufacturing integrated circuits, circuit boards or other electronic or microelectronic devices.

Methods to Verify the Suitability of Candidate Compounds

One wishing to practice the method of the present invention will evaluate candidate compounds for efficacy as OdDHL blockers or BHL analogs. The description below discloses a preferred method of evaluating candidate compounds and products containing the candidate compounds. In brief, one would first wish to grow biofilm in a controlled manner and observe the effect of candidate compounds on the biofilm.

Initial screening will be performed to examine the efficacy of potential cell communication blocking molecules to interfere with biofilm processes that are known to be responsive to regulation by homoserine lactones. These screens are based on information gathered through experimentation on the influence of the acyl homoserine lactone OdDHL on the development of *P. aeruginosa* biofilms. Four parameters have been developed which are sensitive to the presence of HSL compounds.

1. Biofilm average cell cluster depth: This parameter is a measure of the average thickness in microns

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of cell clusters in a biofilm. Depth measurements are performed microscopically, by measuring the maximum distance from the substratum to the apex of a biofilm cell cluster at the bulk water interface in a steady state biofilm for a minimum of 20 cell clusters. Previous measurements have shown that with *P. aeruginosa*, the average cell cluster depth is 102.3 μm with a standard error of the mean of 20.5 μm . When the gene responsible for the production of the homoserine lactone OdDHL is knocked out, the average cell cluster depth drops to only 22.8 μm with a standard error of the mean of 10.0. When OdDHL is added back to the nutrient feed, the biofilm recovers its average cell cluster thickness of 100.1 μm with a standard error of 25.2, demonstrating that the homoserine lactone is responsible for mediating biofilm cell cluster thickness.

2. Biofilm cell nearest neighbor analysis: This parameter is a measure of the average distance spacing between cells within a biofilm cell cluster or between cells at a substratum. Both measurements are influenced by the presence of the homoserine lactone OdDHL. The nearest neighbor analysis is performed by imaging 20 or more fields in a biofilm reactor and electronically determining the centroid of each cell. The relative distances from a marker cell to all other cells is computed and iterated for each cell in the population. The average nearest neighbor is then calculated. Figure 1 shows results from a nearest neighbor analysis performed at the substratum for wild type *P. aeruginosa* (PAO1), and mutants knocked out in the ability to produce OdDHL and BHL (JP2), OdDHL (JP1), and BHL (PDO100). The bar graph demonstrates that the wild type average nearest neighbor is over 9 μm in distance compared to the OdDHL knockout which is under 4 μm . When OdDHL is added to the nutrient feed of the mutant defective in the production of that HSL, the average nearest neighbor increases to just over

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9 um in distance. This demonstrates the influence of cell-cell communication on the arrangement of bacteria in biofilm cell clusters.

3. Effect of detergent on Biofilm dispersion: The detergent SDS has been shown to have no effect on the integrity of cell clusters formed by wild type *P. aeruginosa*. It has been shown previously (Davies, Doctoral Thesis, Montana State University, 1996) that matrix polymer, specifically alginate, prevents the dissolution of *P. aeruginosa* cell clusters treated with 0.2% SDS. However, when cell clusters are formed in the absence of alginate they are completely disrupted in under 30 minutes (Figures 2a, 2b, 2c and 2d). By microscopic examination of cell cluster thickness before and after treatment with SDS or by capturing effluent samples following similar treatment, a quantitative evaluation of the effect of agents capable of blocking the production of detergent resistant matrix material can be made.

4. Alginate promoter reporter activity: The alginate promoters *algC* and *algD* have been shown to be sensitive to the presence of both OddHL and BHL (Figures 3a and 3b). Alginate is a common matrix material in biofilms, and is not exclusive to *P. aeruginosa*. The sensitivity of *P. aeruginosa* to HSL activity provides a rapid assay for HSLs that is sensitive and quantitative. The responsiveness of the specific biofilm genes *algC* and *algD* to HSL activity allows assays to be performed in liquid or on semi solid medium. This provides the opportunity to perform rapid screening of test molecules for their effect on alginate production.

a. Method to grow biofilm in 'slide reactor'.

An autoclavable petri dish is equipped with an inlet tube set into the upper portion of the lid and an outflow tube set within the edge of the lower portion. A microscopy slide is placed inside the Petri dish such that

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the inlet tube is above the upper portion of the slide and the outflow tube at the middle to the lower edge. (The Petri dish and attached tubing are autoclaved and not opened until the biofilm is harvested.) While horizontal, a small amount (e.g. 3 ml) of a suspension of biofilm forming microorganisms (e.g. *Pseudomonas aeruginosa*) is injected through the wall of the tubing so that it drips onto the microscopy slide and covers the slide. After a suitable time, e.g. 1 hour, the Petri dish is raised into a slanted position, so that the inlet tube is at the top and the outflow at the bottom with the microscope slide vertically placed between the two. The slide drains through the outflow. After a suitable drying time (preferably 10 to 30 minutes) the inlet tube is connected via a pump to a reservoir of dilute media, e.g. Tryptic Soy Broth at 1/100 the normal strength. The media is pumped at a slow rate so that 1 drop is dispensed onto the top of the slide approximately every 10 seconds. The drop will run down the slide and exit via the outflow. A biofilm will form on the slide and will be visible after several days. A good time to use the biofilm for testing is when it reaches a total density of 10^6 to 10^9 cells which usually happens after 5 - 6 days. Several such drip reactors may be set up in a parallel fashion.

25 b. Determining whether candidate compounds are BHL analogs by analyzing whether the compounds remove biofilm buildup from surfaces (Laboratory Method).

30 Once biofilm has formed on the slides, three or more slides may be used for treatment, although at least three should be left as untreated controls. Treatment may be done within the Petri dish by switching the inflowing medium to the treatment solution to be tested. The treatment solution will contain a candidate compound or

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